JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Histopathological findings and diagnosis of cutaneous leishmaniasis, confirmed by PCR, in an endemic region of Brazil

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Abstract

Introduction: Diagnosis of cutaneous leishmaniasis (CL) is difficult, and the correct use of histopathological criteria can be useful in clinical practice. The present study evaluates the association between histopathological findings and the results of polymerase chain reaction (PCR) in clinically suspected cases of CL.

Methodology: Skin samples were received in a laboratory from an endemic region of Brazil for over nine years. Associations were analyzed by means of the Chi square test with a 5% level of significance.

Results: Of the 222 examined samples, 190 (85.6%) tested positive by PCR. All 25 cases identified by microscopic examination also tested positive by PCR. Except for the more intense inflammatory infiltrate, all other evaluated histological variables (ulceration, epidermal hyperplasia, hyperkeratosis, presence of granuloma, neutrophils, histiocytes, lymphocytes, plasmocytes, and necrosis) were not significantly associated with PCR positivity.

Conclusions: The intensity of the inflammatory infiltrate is a good indicator of the occurrence of CL. Histopathological aspects are useful to increase the predictive values of CL diagnoses, but PCR is still necessary to confirm or exclude the disease.

Key words: cutaneous leishmaniasis; diagnosis; PCR; histopathology.

J Infect Dev Ctries 2024; 18(6):895-899. doi:10.3855/jidc.18120

(Received 21 February 2023 – Accepted 05 December 2023)

Introduction

Leishmaniasis is a group of diseases that occur in tropical and subtropical areas. They are caused by almost 20 species of protozoa transmitted through the blood meal of infected sandfly females [1]. The complex ecology and epidemiology of leishmaniasis contribute to their propagation [1]. The parasites affect more than 350 million people, with an incidence of 1.3 million new cases annually [2].

The broad spectrum of leishmaniasis can be divided into two main clinical forms: visceral and tegumentary leishmaniasis (TL); which includes cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and disseminated cutaneous leishmaniasis. TL is the most prevalent clinical form of leishmaniasis [3]. Its dissemination raises concerns because it is difficult to diagnose and does not always receive appropriate treatment [4].

CL lesions are characterized by chronicity, latency, painlessness, and destructive potential of the upper airways [5]. Other infectious dermatoses and cutaneous neoplasms are the main differential clinical diagnoses. A valid diagnosis is essential for timely treatment of CL [6].

The main diagnostic tests of CL involve the detection of cellular or humoral immune responses to *Leishmania* infection, in addition to the presence of the parasite itself or its genetic material. The specificity of the tests that detect the immune response is low but they have good sensitivity [7]. The opposite occurs with tests that aim to visualize the parasite. Considering sensitivity and specificity, molecular tests present the best accuracy [3], although they are expensive and not available worldwide.

Studies on the histopathological findings of leishmaniasis have aimed to describe the frequency of histological variables, without performing statistical comparisons in clinically suspected individuals by means of molecular diagnosis [1,6].

Based on the difficulty of diagnosing TL and the high cost of the molecular tests, the present study evaluated the association between histopathological variables and polymerase chain reaction (PCR) results from clinically suspected cases of the disease.

Methodology

This is an observational, cross-sectional study. Skin samples from clinically suspected cases of CL were sent to a Laboratory of Cytology and Pathology at a Brazilian municipality located in an endemic region for the disease. The laboratory had a coverage area of more than 80 cities and more than 1.5 million inhabitants [8]. Specimens were received from 1 January 2006 to 31 December 2015. Clinical information related to age, gender, and location of the lesions was collected from anatomopathological reports. The duration and the evolution of the clinical characteristics of the lesions were not available.

Cuts measuring 3.5 µm from the specimens were stained with hematoxylin and eosin. No special stains specific for *Leishmania* were applied. In cases of granulomatous inflammatory processes, Ziehl-Neelsen and silver stains were used in the search for mycobacteria and fungi, respectively.

In the microscopic analysis, all materials were blindly examined by a dermatopathologist previously trained by the International Committee for Dermatopathology.

The histopathological variables were categorized as: ulceration (present vs. absent), epidermis hyperplasia (present vs. absent), hyperkeratosis (present vs. absent), inflammatory infiltrate (intense, moderate or discrete) [9,10], presence of granulomas (present vs. absent), neutrophils (present vs. absent), histiocytes (present vs. absent), lymphocytes (present vs. absent), plasmocytes (present vs. absent), proliferation of vessels (present vs. absent), or necrosis (present vs. absent). Due to the extensive ulceration, analysis of the epidermis and stratum corneum was not performed on two slides.

Microscopy was considered positive for cases with structures compatible with amastigotes inside the macrophages. At immersion (1000X), amastigote shapes with round or oval morphology were analyzed with sizes between 2 µm and 4 µm, showing nucleus and kinetoplast [11].

The cases where histological examination had conclusive diagnoses for other infectious, neoplastic, or inflammatory skin diseases (e.g. leprosy, histoplasmosis, squamous cell carcinoma, paracoccidioidomycosis, basal cell carcinoma, leprosy, cutaneous tuberculosis, aspergillosis, cryptococcosis, chromomycosis, Behçet's disease, Wegener's granulomatosis, sporotrichosis, seborrheic keratosis, histoplasmosis, granulomatous rosacea, hematopoietic neoplasia, actinic keratosis, sarcoidosis, and unspecific spongiotic dermatitis) were excluded.

After extracting DNA from the lesion-edge fragments, DNA molecule measurements and purity assessments were conducted on all samples. The presence, concentration, and quality of the extracted DNA were evaluated using the Nanodrop®-1000 spectrophotometer. Protein contamination levels were determined by the 260/280nm wavelength ratio, with an absorbance value range of 1.8 to 2. The 260/230nm wavelength ratio was applied in the case of other contaminants, with an absorbance value range of 1.5 to 8. The extraction process involved the use of proteinase K and RNase to ensure sample purity, exclusively preserving DNA. Standard positive controls included *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903), and *L. guyanensis* (MHOM/BR/75/M4147); while ultrapure water was utilized as the negative control.

Primers that flank the conserved region of the *Leishmania* kDNA minicircle were used for PCR: primer A: 5′(C/G)(C/G)(G/C) CC(C/A) CTA T(T/A)T TAC ACC AAC CCC3′ and primer B: 5′GGG GAG GGG CGT TCT GCG AA3′, which amplified a 120 bp fragment. The PCR reaction was prepared for a final volume of 25 µL using the following reagents from Invitrogen (Carlsbad, USA): 2.5 µL buffer (1X), 0.75 µL $MgCl_2$ (1.5 mM), 0.5 µL dNTP mix (200 µM), 0.25 µL of *Taq* platinum® DNA polymerase (1 unit), 1.25 µL of forward primer (0.2 mM) and 1.25 µL of reverse primer (0.2 mM) (Prodimol, Belo Horizonte, Brazil), and 13.5 µL of ultrapure water [12].

Amplification of the template DNA was performed in a thermocycler (ProFlex PCR System - Applied Biosystems, Foster City, USA) with the following amplification cycle: 94 ºC (4 minutes); followed by 30 cycles of 94 ºC (30 seconds) for denaturation, 60 ºC (30 seconds) for annealing, and 72 ºC (30 seconds) for an extension. The final extension was done at 72 ºC for 10 minutes.

The amplification products were separated on agarose gel (1.2%) at 100 volts for 35 minutes. In the end, the gel was stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) and visualized in the photo documentation transilluminator (Life Technologies Carlsbad, USA) to capture and analyze the gel images.

Descriptive statistics analysis, including Chi square tests, were performed to verify the association of histopathological variables with PCR results. A *p* value of < 0.05 was considered significant. The analyses were performed in software R, version 3.5.1.

This study was approved by the Research Ethics Committee of the Federal University of São João Del-Rei (CEPES-UFSJ/CCO), CAAE: 68338817.9.0000.5545).

Results

Of the total 351 samples with suspected cases of leishmaniasis, 129 were excluded after histopathological analysis. There was a high frequency of diagnosis of epithelial neoplasms (squamous cell carcinoma and basal cell carcinoma) and infectious fungal diseases. Among the 222 remaining patients included in the present study, 128 (57.7%) were male and 94 (42.3%) were female, with a median age of 54 years. Lesions were most frequent in the lower limbs (Table 1).

Amastigote shapes compatible with *Leishmania* ssp. were identified in 25 specimens. Upon microscopic analysis (Figure 1), 139 samples (62.61%) presented ulceration, 192 (86.48%) epidermis hyperplasia, 201 (95.04%) hyperkeratosis, 134 (60.36) moderate/intense inflammatory infiltrate, 88 (39.64%) granulomas, 117 (52.7%) neutrophils, 194 (87.38%) histiocytes, 218 (98.19%) lymphocytes, 199 (89.63%) plasma cells, 209 (94.14%) vessel proliferation, 33 (14.86%) vasculitis, and 17 (7.65%) necrosis (Table 2).

Table 1. Demographic and basic characteristics of the disease in patients with clinical suspicion of ATL (N = 222).

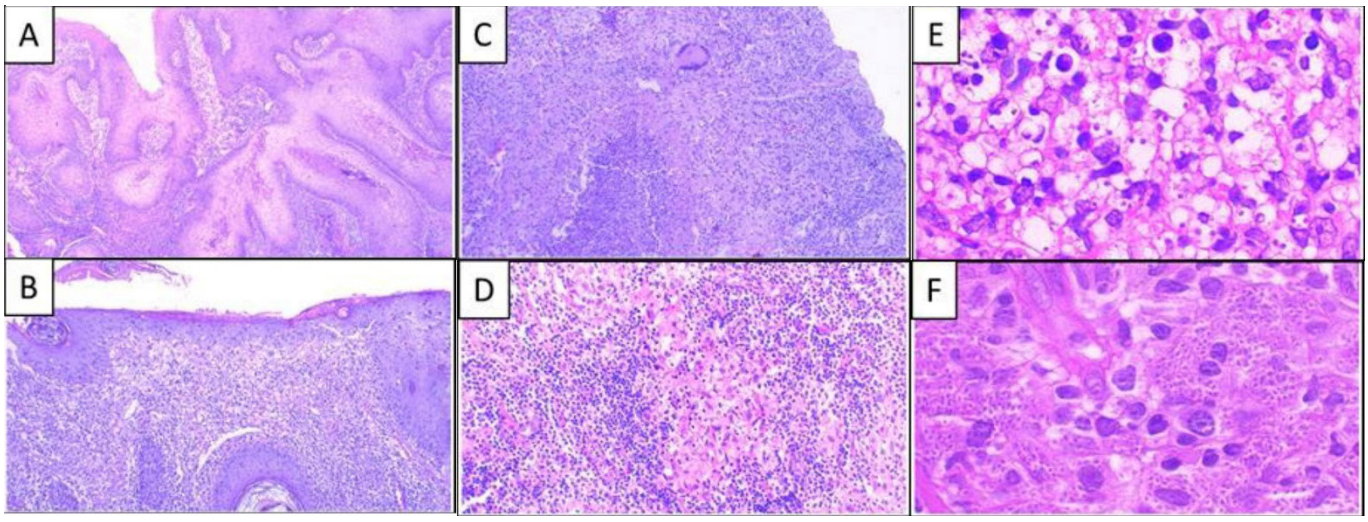
Characteristics	Patients with clinical hypothesis of ATL n (%)
Gender	
Male	128 (57.7)
Female	94 (42.3)
Lesion site	
Head	49 (22)
Trunk	13 (5.8)
Upper limbs	39 (17.7)
Lower members	121 (54.5)
Population age data	
Minimum age	6
Maximum age	96
Amplitude	90
Mean	53
Median	54
SD	19.8
IQR1	39.2
IQR3	67

ATL: American tegumentary leishmaniasis; IQR: interquartile range; SD: standard deviation.

The PCR for detecting *Leishmania* ssp. identified 190 (85.6%) positive and 32 (14.4%) negative cases (Table 2). PCR was positive in all the 25 samples that were identified as positive by histopathological analysis.

The proportion of positive PCR results was significantly higher in samples with intense inflammatory infiltrate. All other evaluated histological variables did not have statistically significant associations with the results of the PCR (Table 2).

Figure 1. Leishmaniasis: **A.** pseudoepitheliomatous hyperplasia (HE 40×); **B.** atrophic epidermic (HE 100×); **C.** chronic granulomatous inflammatory process with giant multinucleated cell (HE 100×); **D.** chronic inflammatory process with granulomatous formation (HE 200×); **E.** amastigote shape within macrophages (moderate parasitic load; HE 1000×); **F.** amastigote shape within macrophages (high parasitic load; HE 1000×).



HE: Hematoxylin and eosin.

Discussion

The present study analyzed a large number of samples of clinically suspected CL cases in an endemic region of Brazil and identified histopathological variables associated with PCR positivity.

Our results corroborate the data previously described in the literature, which indicate a high frequency of intense and mixed inflammatory infiltrate (formed by lymphocytes, histiocytes, and plasmocytes) associated with angiogenesis and ulceration in most cases of leishmaniasis [1]. The identification of higher PCR positivity in samples with intense inflammatory infiltrate is novel and may be useful in clinical practice. However, since the presence of inflammatory infiltrates is not specific to CL, the diagnosis must be complemented with adequate laboratory and clinical-epidemiological investigations.

Although differences in the proportions of positive results were identified in the categories of some variables, none of the other evaluated microscopic findings were significantly associated with the result of molecular analysis. Since leishmaniasis have many clinical presentations (papules, plaques, ulcers, or nodules) depending on the type of parasite, species, and stage of the disease, there is also great variability in histopathological findings [13]. Our results demonstrate the complexity of identifying a specific histological pattern of suspicion.

Our results also indicate the importance molecular methods, such as PCR, in the diagnosis of CL. Our data is similar to the observations of Cardozo *et al.*, who analyzed 360 samples and 325 (90%) presented positive results in PCR [1]. PCR presents high levels of sensitivity and specificity, and good reliability for the diagnosis of leishmaniasis. Thus, its use should be encouraged [14-16].

Another tool for CL diagnosis is immunohistochemical analysis. However, commercially available antibodies have low sensitivity [17]. Recently, the applicability of the CD1a antibody (clone MTB1) for the diagnosis of cutaneous leishmaniasis (CL) in old world samples was demonstrated. However, for new world species, this marker showed only 44% positivity, reinforcing the importance of other types of diagnostic tests [17].

One possible limitation of the study is that the analyzed samples were collected not only by dermatologists, but also by surgeons and general practitioners, without a standard procedure. Furthermore, there was a lack of information regarding the treatment administered to the individuals, potentially impacting the histopathological findings and the detection of amastigotes in the samples. However, it is worth noting that the participants were sourced from similar areas, potentially resulting in minimal variability in terms of medical care. As a result, the overall impact of this limitation on the study's findings is likely to be relatively minor. Finally, as the number of negative PCR cases was low, the analysis had reduced statistical power. Nevertheless, we emphasize

Table 2. Associations of histopathological variables and PCR in clinically suspected cases of cutaneous leishmaniasis.

Variable		PCR− n (%)	PCR+ n (%)	χ^2 *p* value
Epidermis hyperplasia	No	5 (17.9)	23 (82.1)	0.595
	Yes	27 (14.1)	165 (85.9)	
Histiocytes	No	2 (7.1)	26 (92.9)	0.241
	Yes	30 (15.5)	164 (84.5)	
Hyperkeratosis	No	3 (33.3)	6 (66.7)	0.103
	Yes	29 (13.7)	182 (86.3)	
Lymphocytes	No	1 (25.0)	3 (75.0)	0.543
	Yes	31 (14.2)	187 (85.8)	
Necrosis	No	30 (14.6)	175 (85.4)	0.746
	Yes	2 (11.8)	15 (88.2)	
Neutrophils	No	17 (16.2)	88 (83.8)	0.475
	Yes	15 (12.8)	102 (87.2)	
Plasmocytes	No	5 (21.7)	18 (78.3)	0.291
	Yes	27 (13.6)	172 (86.4)	
Presence of granuloma	No	17 (12.4)	120 (87.6)	0.280
	Yes	15 (17.6)	70 (82.4)	
Proliferation of vessels	No	3 (23.1)	10 (76.9)	0.359
	Yes	29 (13.9)	180 (86.1)	
Ulceration	No	15 (18.1)	68 (81.9)	0.231
	Yes	17 (12.2)	122 (87.9)	
Variation of inflammatory infiltrate	Intense	4 (6.0)	63 (94.0)	0.048 *
	Moderate	11 (16.4)	56 (83.6)	
	Discrete	17 (19,3)	71 (80,7)	
Vasculitis	No	29 (15.3)	160 (84.7)	0.321
	Yes	3 (9.1)	30 (90.9)	

PCR: polymerase chain reaction; χ^2: Chi-square test; * statistically significant association (*p* value < 0.05).

that the size of our population is larger than that of most previous studies and that the procedures (PCR and histopathology) were performed with strict quality control to minimize the occurrence of bias.

Conclusions

Among the histopathological criteria evaluated, only the variation of the inflammatory parameters was associated with a greater chance of a positive molecular results. Thus, excluding cases corresponding to the possible differential diagnoses, and when the amastigote forms are not identified, the intensity of the inflammatory infiltrate can be a good indicator of the occurrence of CL. However, PCR is still indicated to exclude leishmaniasis.

Funding

This study was partially funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; grant code 001).

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Conflict of interests: No conflict of interests is declared.